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BMP-4 Inhibits Neural Differentiation of Murine Embryonic Stem Cells

Michael F. A. Finley, Sandeep Devata, James E. Huettnner

Department of Cell Biology and Physiology and Program in Neuroscience, Washington University Medical School, 660 South Euclid Avenue, St. Louis, Missouri 63110

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ABSTRACT: Members of the transforming growth factor- β superfamily, including bone morphogenetic protein 4 (BMP-4), have been implicated as regulators of neuronal and glial differentiation. To test for a possible role of BMP-4 in early mammalian neural specification, we examined its effect on neurogenesis in aggregate cultures of mouse embryonic stem (ES) cells. Compared to control aggregates, in which up to 20% of the cells acquired immunoreactivity for the neuron-specific antibody TuJ1, aggregates maintained for 8 days in serum-free medium containing BMP-4 generated 5- to 10-fold fewer neurons. The action of BMP-4 was dose dependent and restricted to the fifth through eighth day in suspension. In addition to the reduction in neurons, we observed that ES cell cultures exposed to BMP-4 contained fewer cells that were immunoreactive for glial fibrillary acidic protein or the HNK-1 neural antigen. Furthermore, under phase contrast, cultures prepared from

BMP-4-treated aggregates contained a significant proportion of nonneuronal cells with a characteristic flat, elongated morphology. These cells were immunoreactive for antibodies to the intermediate filament protein vimentin; they were rare or absent in control cultures. Treatment with BMP-4 enhanced the expression of the early mesodermal genes *brachyury* and *tbx6* but had relatively little effect on total cell number or cell death. Coapplication of the BMP-4 antagonist noggin counteracted the effect of exogenous BMP-4, but noggin alone had no effect on neuralization in either the absence or presence of retinoids. Collectively, our results suggest that BMP-4 can overcome the neuralizing action of retinoic acid to enhance mesodermal differentiation of murine ES cells. © 1999 John Wiley & Sons, Inc. *J Neurobiol* 40: 271–287, 1999

Keywords: induction; retinoic acid; serum-free; TuJ1; noggin

Bone morphogenetic proteins (BMPs) are secreted proteins (Hogan, 1996) that regulate cellular differentiation in a wide variety of tissues, including kidney, lung, and brain. Developmental studies of cultured cells from the mammalian nervous system have revealed a number of distinct actions for BMPs that depend on both the source and the age of the target cells (reviewed in Mehler et al., 1997). For example, BMP-2 and -4 stimulate neurogenesis in cultured neural crest stem cells (Shah et al., 1996), regulate transmitter phenotype in sympathetic neurons (Fann and

Patterson, 1994), promote glial differentiation of progenitor cells from the telencephalic subventricular zone (Gross et al., 1996), and help to establish dorsal-ventral polarity in the spinal cord (Liem et al., 1997).

In contrast to this work on relatively mature embryonic and postnatal neurons, much less is known about the role of BMPs at the very earliest stages of mammalian neurogenesis. During gastrulation, BMP-2 and -4 mRNA is found in the primitive streak and in localized regions of early embryonic mesoderm and neural tissue (Jones et al., 1991; Winnier et al., 1995). To address BMP-4 function in mammalian development, mutant mice have been generated that lack BMP-4 (*bmp-4*) or its receptor (*bmpr*) (Winnier et al., 1995; Mishina et al., 1995). Most embryos that are homozygous null for *bmp-4* or *bmpr* fail to gas-

Correspondence to: J. E. Huettnner

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trulate owing to the loss of early mesoderm. Consistent with these observations, exposure to BMP-4 and other transforming growth factor- β (TGF- β) family members can enhance the production of mesoderm during *in vitro* differentiation of embryonic stem (ES)

cells (Johansson and Wiles, 1995). These results argue strongly that BMP-4 is involved in early mesodermal differentiation, but leave open the question of its role in early mammalian neural induction.

Evidence that BMPs may negatively regulate neu-

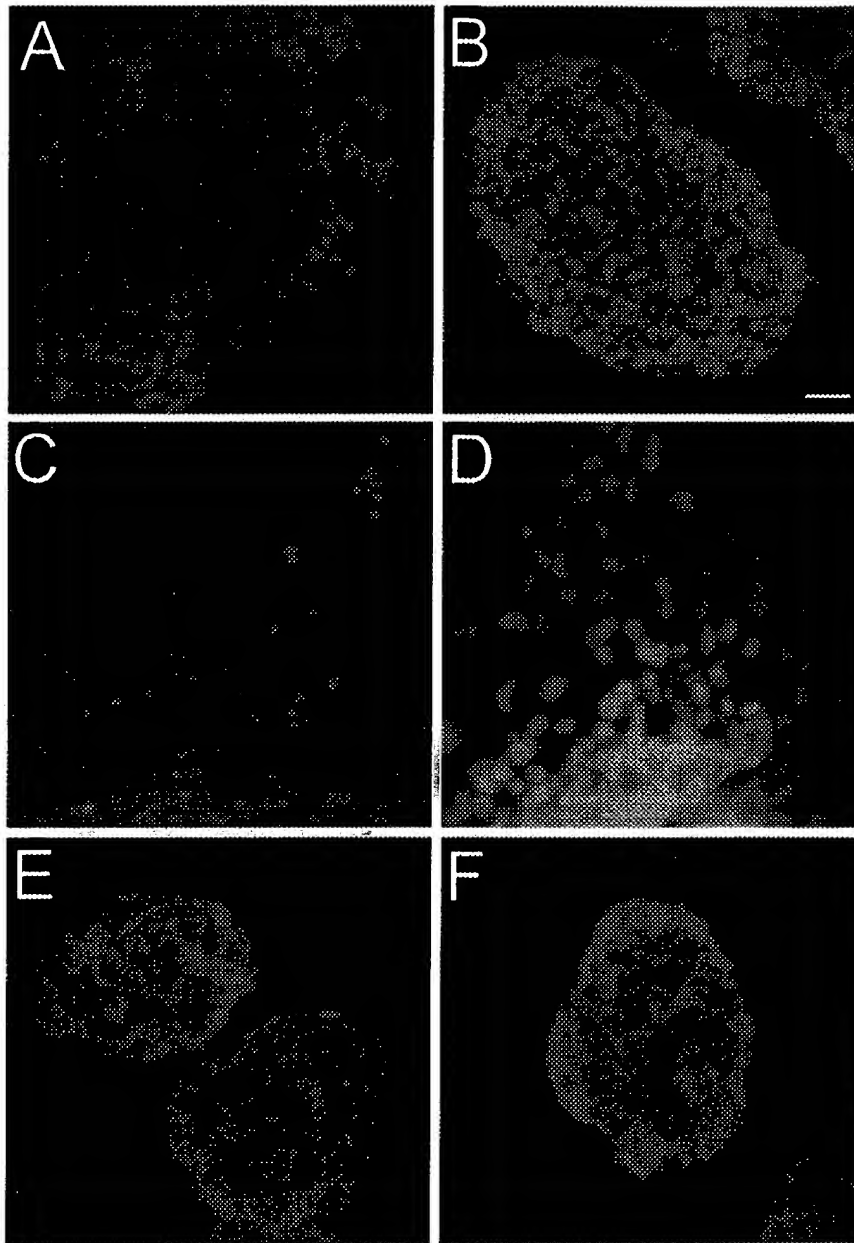


Figure 1 Bone morphogenetic protein-4 inhibits neuronal differentiation. (A–D) Expression of neuron-specific class III beta-tubulin was visualized by indirect immunofluorescence in sections through control (A) and BMP-4-treated (B) 8-day embryoid bodies, and in plated cultures 5 days later [(C), control; (D), BMP-4-treated]. DAPI-stained nuclei are shown in blue, and immunofluorescence in red. Immunofluorescence for a neuron-specific nuclear antigen recognized by the NeuN antibody is visualized in sections of 13-day control (E) and BMP-4-treated (F) embryoid bodies. BMP-4 was applied continuously at 8 ng/mL in (B,D) and between days 5 and 8 in (F). Scale bars: 34 μ m (A,B), 30 μ m (C,D), 50 μ m (E,F).

ral induction has come from a series of studies on *Xenopus* embryos. In *Xenopus* ectoderm, BMP-4 induces epidermis and inhibits neural differentiation (reviewed in Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997). Moreover, neuralizing molecules that originate from Spemann's organizer, including noggin, chordin, and follistatin, appear to act by binding and inactivating BMP-4 (Zimmerman et al., 1996; Piccolo et al., 1996; Fainsod et al., 1997). These data suggest that in *Xenopus* neuralization is a default fate of the ectoderm, which is inhibited by BMP-4 but allowed to occur in the presence of BMP-4 antagonists such as noggin or chordin (Hemmati-Brivanlou and Melton, 1997).

To address the role of BMPs in the control of mammalian neural specification, we investigated the action of BMP-4 on neural induction of ES cells, the totipotent cells that are used to generate transgenic mice (Martin, 1981; Evans and Kaufman, 1981). Previous work (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Okabe et al., 1996) has shown that aggregation of ES cells into embryoid bodies (Martin and Evans, 1975; Coucouvanis and Martin, 1995) followed by stimulation with all-*trans*-retinoic acid leads approximately 35–40% of the cells to acquire a neuronal phenotype, as judged by their expression of neuron-specific markers and characteristic electrophysiological properties (Bain et al., 1995; Finley et al., 1996). An additional proportion of the cells adopt a glial phenotype as determined by immunoreactivity for glial fibrillary acidic protein (GFAP). In the present study, we demonstrate a dramatic reduction in the generation of neurons and glial cells and an increase in mesodermal differentiation, following exposure of embryoid bodies to BMP-4 during a specific time period in their differentiation. This action of BMP-4 could be overcome by coincubation with an excess of the protein noggin (Lamb et al., 1993); however, treatment with noggin alone caused no significant change in the proportion of cells that acquired a neuronal phenotype. Our results indicate that BMP-4 inhibits mammalian neuralization primarily by directing ES cells toward a mesodermal fate.

MATERIALS AND METHODS

ES Cell Culture and Induction

The D3 line of ES cells (ATCC No. CRL-1934) was propagated in Dulbecco's modified Eagle's medium (DMEM) containing 20% calf serum, nucleosides (30 μ M adenosine, cytosine, uridine, guanine, and 10 μ M thymidine), leukemia inhibitory factor (LIF) (1000 U/mL ESGRO; Life Technologies), and 0.1 mM 2-mercaptoethanol. Cells were passaged every 2–3 days. Methods for serum-free induction

were based on the 4-/4+ protocol of Bain et al. (1995). Confluent cells were harvested with protease XXIII (Sigma), collected by centrifugation (70 \times g for 5 min), and resuspended in neurobasal medium with B27 supplement (NB+B27; Life Technologies). B27 supplement includes retinyl acetate (Brewer et al., 1993), which can substitute for retinoic acid to promote neuralization (manuscript in preparation). Approximately $2-4 \times 10^6$ cells were transferred to a 6-cm petri dish coated with 1.5 mg/mL agarose, in which they remained suspended and formed aggregates. Aggregates were maintained for 8–13 days in NB+B27. For some experiments, B27 supplement lacking retinyl acetate [B27(-)] substituted for B27. In these cases, RA (0.5–1 μ M; Sigma) was added during the fifth through eighth day of aggregation. Both of these serum-free induction conditions produced essentially equivalent levels of neural differentiation to that observed in the original 4-/4+ protocol. For most experiments, aggregates were plated onto gelatin-coated petri dishes after 8 days in suspension. Within 5 days of plating, neurons and glia migrated out of the aggregates.

Bone morphogenetic protein-4 and recombinant human (rh) Noggin were prepared as stock solutions in neurobasal medium plus 0.1% bovine serum albumin (BSA) and stored at -80°C in 20- μ L aliquots at 4 and 16 μ g/mL, respectively. Growth media for all experiments received 0.1% BSA (Sigma) with or without added factors. BMP-2, -4, -6, and -7 were kindly provided by Dr. Rod Riedel at the Genetics Institute. Dr. Aris Enconomidis at Regeneron generously provided BMP-4, rhNoggin, and rhNoggin Δ B2.

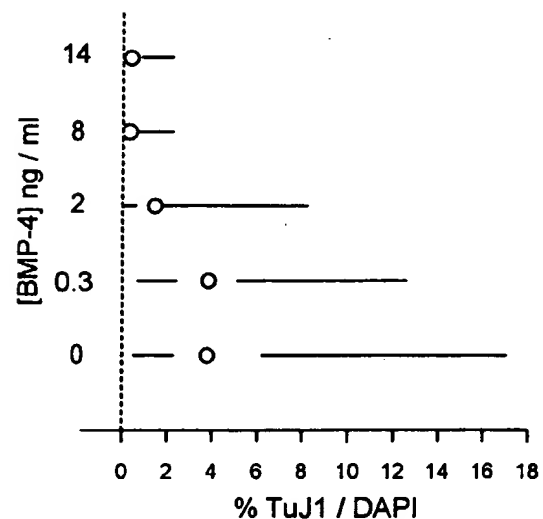


Figure 2 Inhibition of neuronal differentiation by BMP-4 is dose-dependent. Quartile plots show the percentage of TuJ1-positive cells in 8-day aggregate sections. Points indicate the median values; lines span the lower and upper 25% of the distribution. At least 20 sections were analyzed for each concentration from one (0.3, 2, and 14 ng/mL) or two (0 and 8 ng/mL) separate inductions. Increasing concentrations of BMP-4 produce significant inhibition of TuJ1 expression ($p < .001$ for 2, 8, and 14 ng/mL relative to control, Mann-Whitney rank sum test).

The $\Delta B2$ mutation reduces binding to heparin relative to wild-type rhNoggin, but does not prevent interaction with BMP-4 (A. Economides, personal communication). B27 supplement without retinyl acetate, B27(-), was generously donated by Dr. Paul Price at Life Technologies.

Immunofluorescence of Embryoid Bodies or Cultured Cells

Aggregates or cultures were fixed for 20 min at room temperature with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4. After four rinses with phosphate-buffered saline (PBS), aggregates were equilibrated with 30% sucrose and sectioned (5–10 μ m) on a cryostat. Sections were incubated overnight at 4°C with primary antibodies. After rinsing four times with PBS, sections were incubated for 1 h at room temperature with Cy3-conjugated secondary antibodies (Chemicon, Temecula, CA). Dilutions for primary antibodies to specific antigens were as follows: class III β -tubulin (TuJ1, 1:6000; kindly provided by A. Frankfurter), NeuN (monoclonal

supernatant 1:200; kindly provided by R. J. Mullen), GFAP (1:800; Sigma), and HNK-1 (monoclonal supernatant 1:5; Beckton Dickinson; kind gift of J. Sanes). For the following antibodies, cultures were fixed in MeOH or MeOH-acetic acid (1:1) at -20°C for 20 min. Dilutions were as follows: keratin (1:100, Sigma); muscle-specific actin (HUC1-1; 1:500, ICN); and 1:5 for vimentin, osteopontin, stro-1, MJ7/18, and MECA-32 (all obtained from the Developmental Studies Hybridoma Bank as monoclonal supernatants). Sections and cultures were counterstained with DAPI to visualize nuclei of all cells.

Cell Proliferation and Apoptosis

Two methods were used to measure cell proliferation. The CyQUANT Cell Proliferation Assay (Molecular Probes) relates total DNA content to cell number. For each experiment, we generated a standard curve of fluorescence versus cell number based on serial dilutions of known numbers of undifferentiated ES cells. Fluorescence of test samples was compared to the standard curve to determine total cell

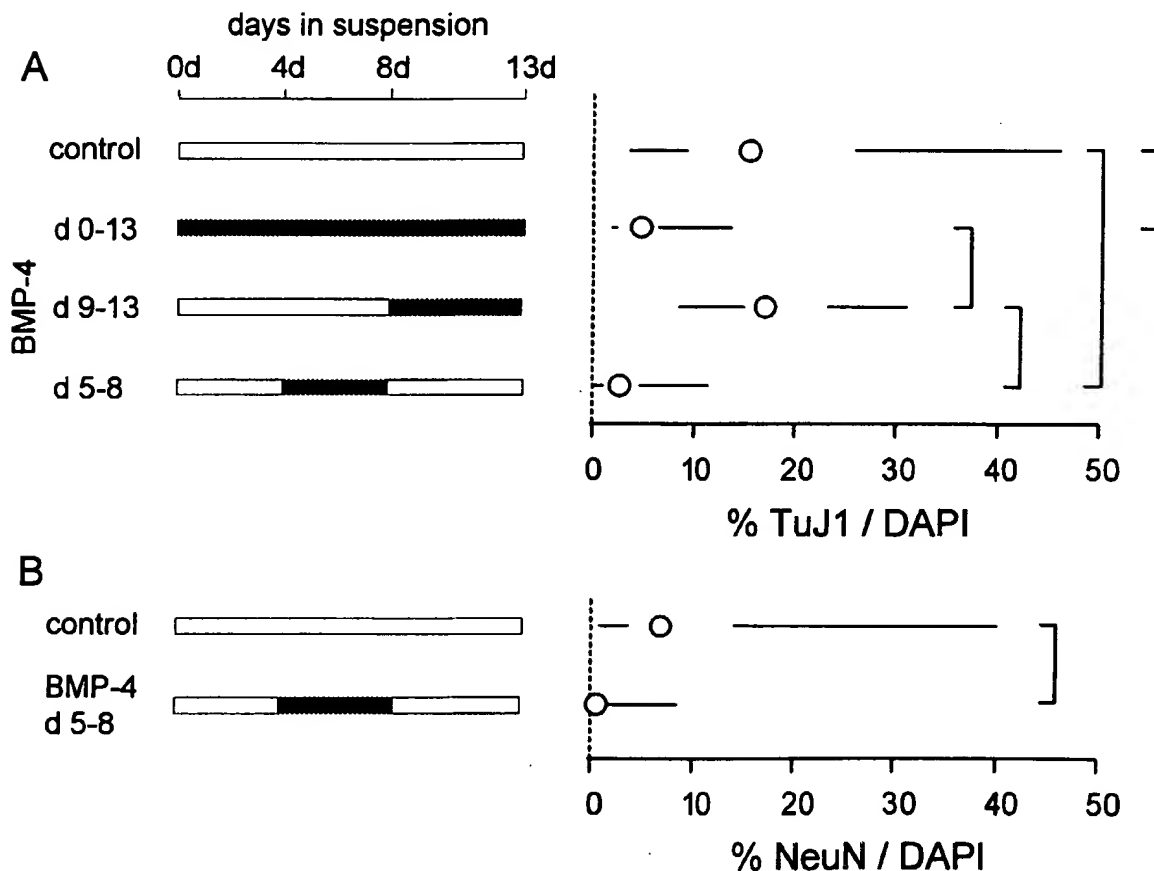


Figure 3 Bone morphogenetic protein-4 acts during a restricted time window. (Left) Period of exposure to BMP-4 (8 ng/mL) during the 13 days in suspension is indicated by the gray bars. (Right) Quantile plots show the percentage of cells stained for TuJ1 (A) or NeuN (B) in aggregate sections. Pooled data from two inductions for each condition are shown. Pairwise comparisons indicated by brackets are significantly different ($p < .001$, Mann-Whitney rank sum test). (See Table 1.)

number. For each induction, equal numbers of ES cells were seeded into nonadhesive, six-well culture plates in NB+B27 with or without BMP-4 (8 ng/mL). At each time point, an entire well of aggregates from each condition was harvested by brief centrifugation. The cell pellet was frozen at -70°C and thawed by addition of CyQUANT-GR lysis buffer (1 mL). Fluorescence emission at 520 nm was determined with excitation at 480 nm.

Bromodeoxyuridine (BrdU; Sigma) was added to ES cell aggregates at 3 $\mu\text{g}/\text{mL}$ for 1 h prior to fixation (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M NaPO_4 , pH 7). Aggregates were sectioned, postfixed in MeOH (2×10 min), and stored overnight at -70°C . Sections were rehydrated in PBS, then treated with 2N HCl at 37°C for 1 h. Borate buffer (55% 0.2 M boric acid plus 45% 0.05 M borax, 2×10 min) was used to neutralize the acid. After three PBS rinses, sections were blocked with 0.1% BSA in PBS for 30 min, then incubated for 2 h at room temperature with mouse anti-BrdU (1:200; Boehringer Mannheim) diluted in PBS containing 1 mg/mL BSA. Following PBS wash, Cy3-conjugated goat anti-mouse (1:400; Chemicon) was added for 1 h at room temperature. Sections were rinsed and counterstained with DAPI.

Aggregate sections from control and BMP-4-treated conditions were processed to detect fragmented DNA using the ApopTag Plus assay (Oncor, Gaithersburg, MD). Aggregates were fixed with 4% paraformaldehyde and post-fixed, after sectioning, with ethanol/acetic acid (2:1). Sections were rinsed with PBS and incubated with terminal deoxynucleotidyl transferase and digoxigenin-labeled nucleotides for 1 h at 37°C . After a brief rinse, sections were incubated with fluorescein-conjugated anti-digoxigenin for 1 h, rinsed again, and counterstained with DAPI. Labeling was visualized by epifluorescent illumination, and images were acquired on a digital camera to facilitate quantitation. BAF was a gift of Dr. Eugene Johnson.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Ribonucleic acid was prepared from ES cell cultures and reverse-transcribed using the Promega RNAsagents and Reverse Transcription Systems. PCR was performed (PCR Core System; Promega) using the following protocol: 1×60 s at 96°C , 35×15 s at 96°C , 15 s at 50°C , and 60 s at 72°C ; and $1 \times 72^{\circ}\text{C}$ for 7 min. Primer sequences for *brachyury* (Larue et al., 1996) and *GluR6* (Ray and Gottlieb, 1993) were obtained from published sources and synthesized by the Nucleic Acid Chemistry Lab at Washington University. The following primers were used for *GAPDH* based on sequences for mouse *GAPDH* (Sabath et al., 1990; GenBank Accession No. M32599) to generate a 713-bp band: (upstream) 5'-GAGGCCGGTGCTGAGTATGTC-3'; (downstream) 5'-TCCACCACCCTGTTGCTGTAG-3'. Primers for detecting *Tbx6* expression as a 429-bp band were based on the sequence from Chapman et al., 1996 (GeneBank Accession No. U57331): (upstream) 5'-GCCCAGCCCCCTACTCAG-3'; (downstream) 5'-CTCCCTCCATTGCGACTAA-3'.

RESULTS

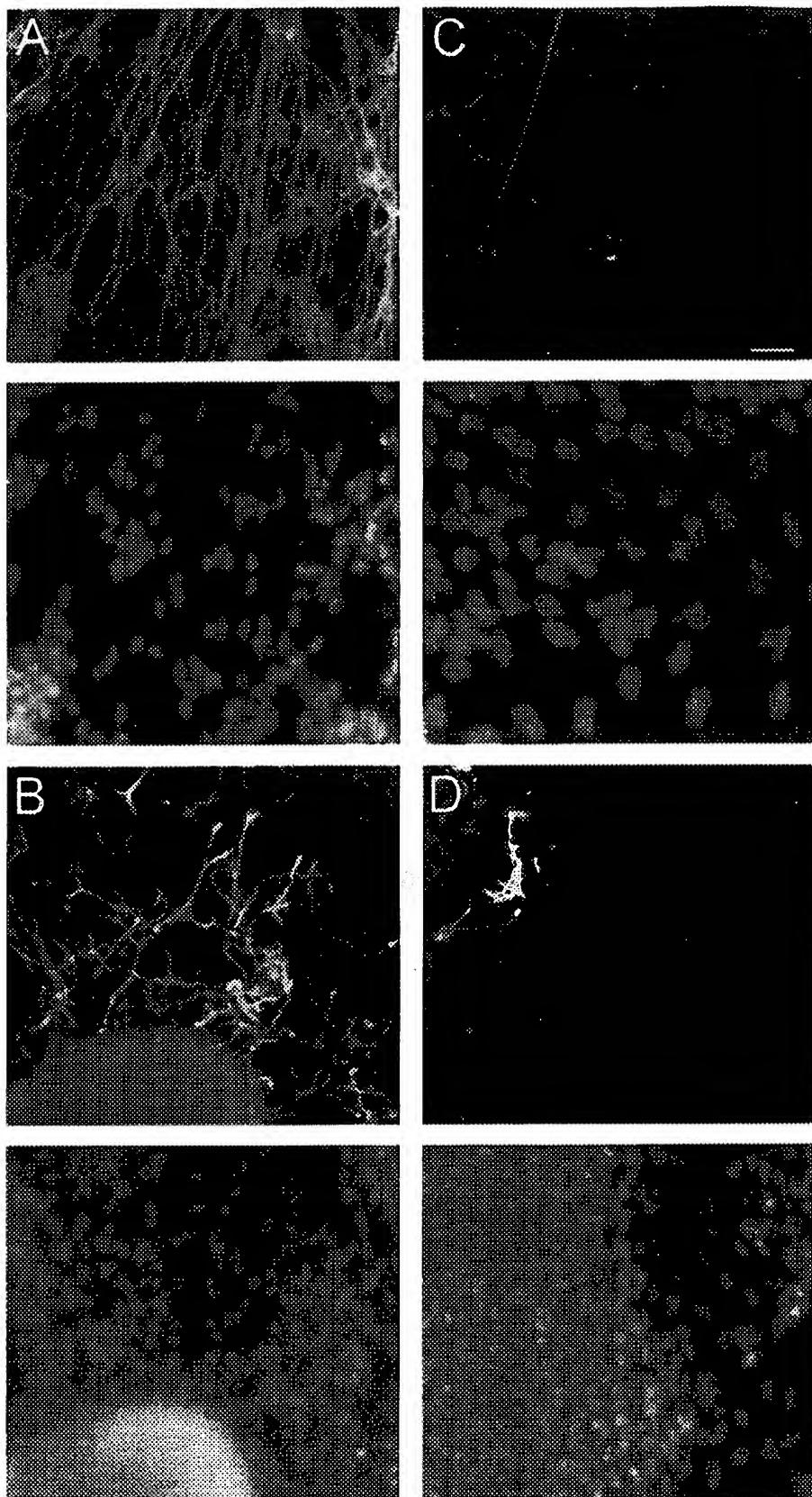
BMP-4 Inhibits Neural Differentiation

To examine the action of growth and differentiation factors on ES cell induction, we first established conditions for efficient production of neurons in a serum-free defined medium. Embryoid bodies were cultured for 8 days in neurobasal medium plus B27 supplements (NB+B27) (Brewer et al., 1993) and then plated onto a gelatin-coated plastic substrate. Under these conditions, ES aggregates are exposed to a precursor of retinoic acid, retinyl acetate, which promotes efficient neuralization (manuscript in preparation). In initial experiments, cultures were scored 5 days after plating for the presence or absence of neuron-like cells observed under phase-contrast optics. To confirm that these cells were indeed neurons, immunoreactivity for the TuJ1 antibody, which recognizes a neuron-specific form of class III β -tubulin (Lee et al., 1990), was visualized in sections of 8-day aggregates and in cultures of plated aggregates. As shown in Figure 1, TuJ1 stained a significant proportion of the cells in 8-day control aggregates [Fig. 1(A)] and revealed a dense network of neurons in cultures of control aggregates 5 days after plating

Table 1 Inhibition of Neurogenesis Depends on Period of Exposure to BMP-4

Exposure to BMP-4 (days)			TuJ1 Expression	n
1-4	5-8	9-13		
Control (retinyl acetate)			+++	20
****	****	*****	+/-	3
****	****		+/-	5
****			+++	3
	****		+/-	16
		*****	+++	2
Control (retinoic acid)			+++	5
	****		+/-	5
****	****	(BMP-2)	+/-	3
****	****	(BMP-6)	+++	1
****	****	(BMP-7)	+++	3

(Left) Exposure to BMP-4 (8 ng/mL) during the three time blocks is indicated by asterisks. (Right) The level of TuJ1 (and/or other neural markers) is scored on a qualitative scale ranging from +/- for a poor neuronal induction [e.g., Fig. 1(B)] to +++ for a strong induction [a high percentage of neurons, e.g., Fig. 1(A)]. n = number of inductions observed. For the first six conditions, aggregates were cultured in neurobasal medium plus B27 supplement containing retinyl acetate. The next two conditions employed B27 supplement that lacked retinyl acetate, but retinoic acid was added for days 5-8 to induce neural differentiation. The last three conditions present the level of neuralization for aggregates maintained in NB+B27 with BMP-2, BMP-6, or BMP-7. Aggregates were exposed to 24 ng/mL BMPs from day 1 through 8, then plated in NB+B27 and analyzed 5 days later.



[Fig. 1(C)]. In contrast, a much smaller fraction of cells was labeled in aggregates that had been exposed to 8 ng/mL BMP-4 throughout the 8-day induction period [Fig. 1(B)]. Moreover, cultures prepared from aggregates treated with BMP-4 contained very few TuJ1-positive cells with neuronal morphology [Fig. 1(D)].

To quantitate the action of BMP-4, we counted the number of TuJ1(+) cells in cryostat sections of control and BMP-4-treated 8-day or 13-day aggregates and plotted the results (Figs. 2 and 3) as a percentage of total cell number. Because acquisition of TuJ1 immunoreactivity occurs as neural precursors begin differentiating into neurons (Lee et al., 1990; Menezes and Luskin, 1994; Memberg and Hall, 1995), this approach should provide a good estimate of the number of cells that have committed to a neuronal phenotype. As shown in Figure 2, exposure to BMP-4 produced nearly a 10-fold reduction in the percentage of cells that expressed high levels of TuJ1 immunoreactivity at 8 days. Immunofluorescent visualization of the neuron-specific nuclear antigen recognized by the NeuN antibody (Mullen et al., 1992) confirmed the substantial reduction in the proportion of neurons in aggregates exposed to BMP-4 [Figs. 1(E,F) and 3].

Concentration Dependence

The action of BMP-4 on ES cell embryoid bodies increased monotonically with concentration (Fig. 2). Inhibition of neural differentiation was not observed with BMP-4 concentrations below 0.3 ng/mL. The effect was half-maximal in the range of 2–8 ng/mL and saturated at concentrations above 14 ng/mL (Fig. 2). These concentrations are in line with previous work on the effects of BMP-4 in other systems (Shah et al., 1996; Gross et al., 1996; Suzuki et al., 1997a). Even with continuous exposure to maximal doses of BMP-4, however, we always observed a few TuJ1-positive cells (Fig. 1), which appeared to be neurons under phase-contrast microscopy. Whole-cell recordings from five of these neuron-like cells in cultures prepared from BMP-4-treated aggregates revealed that all five expressed voltage-gated currents similar to those previously recorded (Bain et al., 1995) from

ES-derived neurons in control cultures (data not shown).

In addition to BMP-4, we tested the effect of other related TGF- β family members on ES cell neuralization (Table 1). BMP-2 was able to inhibit TuJ1 and NeuN expression, although higher doses than used for BMP-4 (24 ng/mL) were required to achieve equivalent inhibition. These observations are consistent with the high sequence homology (Wozney et al., 1988) and overlapping functions of BMP-2 and BMP-4 in some systems (Graham et al., 1994; Shah et al., 1996). In contrast, exposure to BMP-6 or BMP-7 produced no detectable change in the proportion of TuJ1-positive neurons at concentrations up to 24 ng/mL (Table 1).

BMP-4 Acts in a Restricted Time Window

To gain insight into the cellular mechanisms underlying BMP-4's action on ES cells, we examined whether continuous exposure to BMP-4 was required to inhibit neuronal differentiation, or whether treatment with BMP-4 over a more limited time period was sufficient. As shown in Table 1, incubation with BMP-4 (8 ng/mL) for the first 8 days of aggregation produced strong inhibition of neuronal differentiation that did not reverse when cells were subsequently cultured for 5–10 days in medium without BMP-4. Exposure to BMP-4 for only the first 4 days of aggregation, however, had no significant effect on TuJ1 expression (Table 1). This result indicates that BMP-4 does not irreversibly alter undifferentiated ES cells in a manner that prevents them from becoming neurons.

Between days 8 and 13, a large percentage of ES cells underwent differentiation as indicated by the increase in TuJ1 expression over this period (compare Figs. 2 and 3). Exposure to BMP-4 after 8 days of induction (Fig. 3 and Table 1) failed to inhibit expression of neuronal markers and neuron-like morphology, which indicates that BMP-4 does not kill differentiated (TuJ1-positive) neurons and is unlikely to kill the immediate precursors of differentiating neurons. In contrast, BMP-4 treatment from the fifth through eighth day of aggregation was as effective as continual exposure to BMP-4 in reducing expression of

Figure 4 Bone morphogenetic protein-4 reduces expression of HNK1 (A,C) and GFAP (B,D). ES cells were cultured as aggregates in NB plus B27(–) for 8 days. Between days 5 and 8, RA (1 μ M) (A,B) or RA (1 μ M) plus BMP-4 (8 ng/mL) (C,D) was added (cf. Table 1). On day 8, aggregates were plated and cultured for 8 additional days (16 days total) before fixing and staining. DAPI-stained nuclei are shown on the bottom, and immunofluorescence on the top of each lettered panel. Scale bars: 25 μ m (A,C) and 33 μ m (B,D).

TuJ1 and NeuN (Fig. 3). Less complete inhibition was observed following briefer exposure to BMP-4 within this period (not shown). Thus, the fifth through eighth days represent a window of efficacy during which BMP-4 can block the subsequent production of neurons. Interestingly, this is the same time period when retinoic acid is required to induce neuronal differentiation in ES cells (Bain et al., 1995, 1996). As shown in Table 1, we found that BMP-4 was equally effective over this period whether neurogenesis was in-

duced by continuous incubation with retinyl acetate or by coapplication of retinoic acid during this same 4-day window.

BMP-4 Induces Cells of Mesodermal Lineage

When aggregates that had been treated with an effective dose of either BMP-4 or BMP-2 were plated onto an adhesive substrate, a population of cells with a

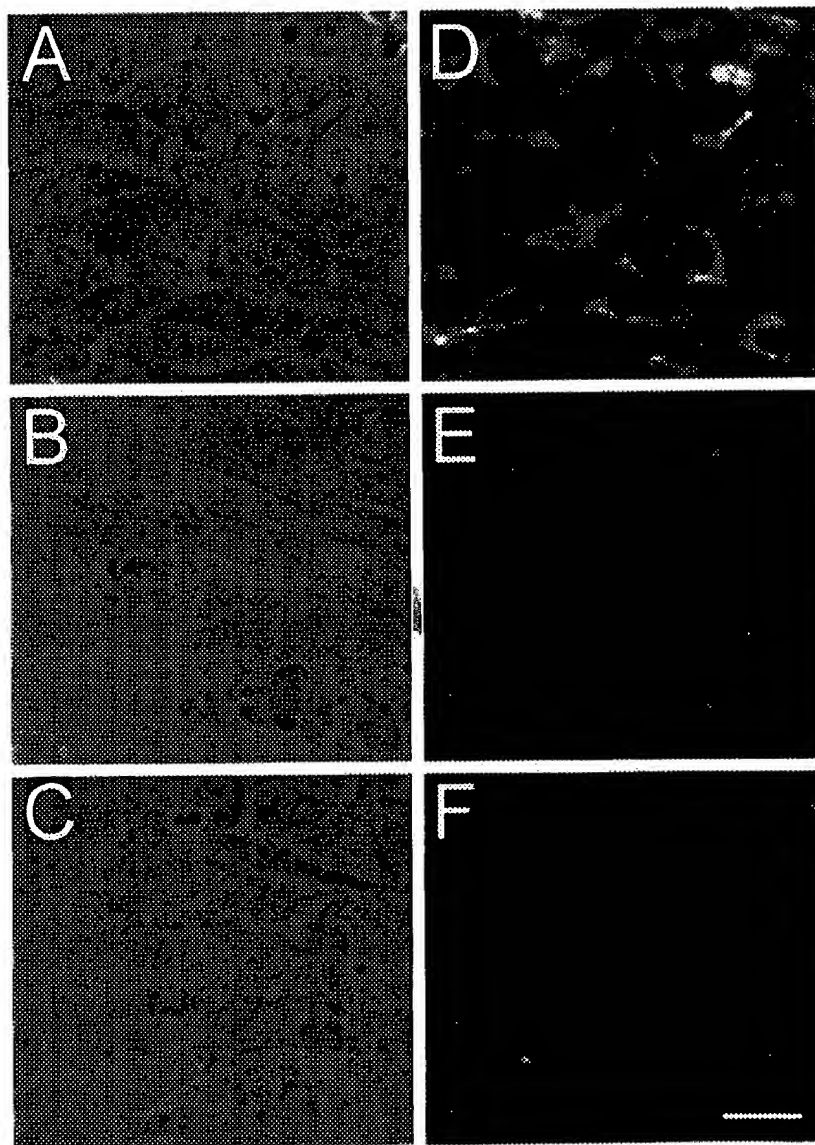


Figure 5 Bone morphogenetic protein-4 promotes differentiation of vimentin-positive cells. (A–C) Phase-contrast images of the flat, elongated cells in cultures from BMP-4-treated (8 ng/mL; days 5–8) aggregates. Embryoid bodies were cultured in NB plus B27 for 8 days (A,B) or NB plus B27(–) for 8 days with 1 μ M RA added for days 5–8 (C), then plated and cultured for 8 additional days before fixation. Photomicrographs of the same fields showing immunofluorescence for vimentin (D), keratin (E), and GFAP (F). Scale bar: 20 μ m.

distinctive flat, elongated morphology migrated out to form confluent patches surrounding the aggregates. This observation suggested that BMP-4 might be promoting a change in fate of the differentiating ES cells. To examine this possibility, we visualized the distribution of several different cell type-specific antigens in cultures prepared from control and BMP-4-treated aggregates. Figure 4 shows immunofluorescent staining with antibodies to GFAP and with the HNK-1 antibody, which recognizes neuroectodermal derivatives (Holley and Yu, 1987). Staining with both of these antibodies was reduced in cultures from aggregates that had been exposed to BMP-4, suggesting an inhibition of both glial and neuronal differentiation.

Importantly, the elongated nonneuronal cells which were prevalent in cultures from BMP-4-treated aggregates did not stain with either of these antibodies. Further testing with additional antibodies revealed

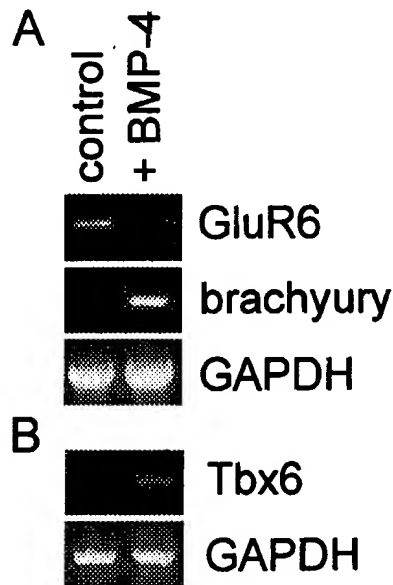


Figure 6 Bone morphogenetic protein-4 induces brachyury and *tbx6* expression. ES cells were aggregated in NB plus B27 for 8 days, then plated and cultured for 7 days before extracting total RNA. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described in Materials and Methods. Exposure to BMP-4 (8 ng/mL, days 5–8) elicited expression of *brachyury* (A) in three of six experiments, and *tbx6* (B) in two of four experiments. Both inductions expressing *tbx6* also expressed *brachyury*. GAPDH expression was similar in both conditions, whereas GluR6 was reduced by treatment with BMP-4. In two of the remaining experiments *brachyury* expression was not detected on days 5 and 6 after plating, whereas low expression was observed in both control and BMP-4-treated cultures in the sixth experiment. Identity of the *brachyury* PCR fragment was confirmed by DNA sequencing.

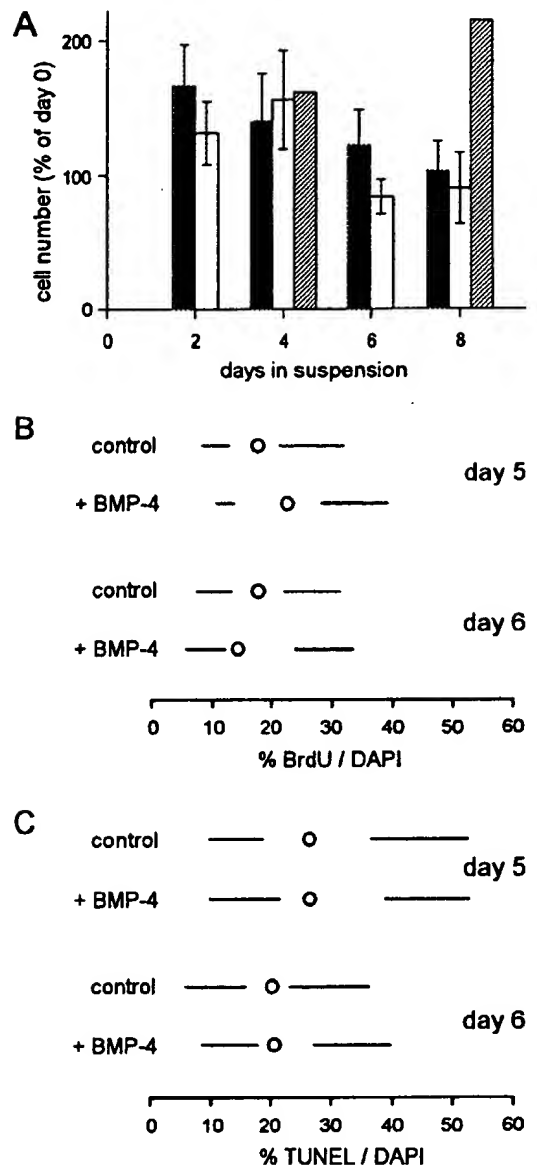
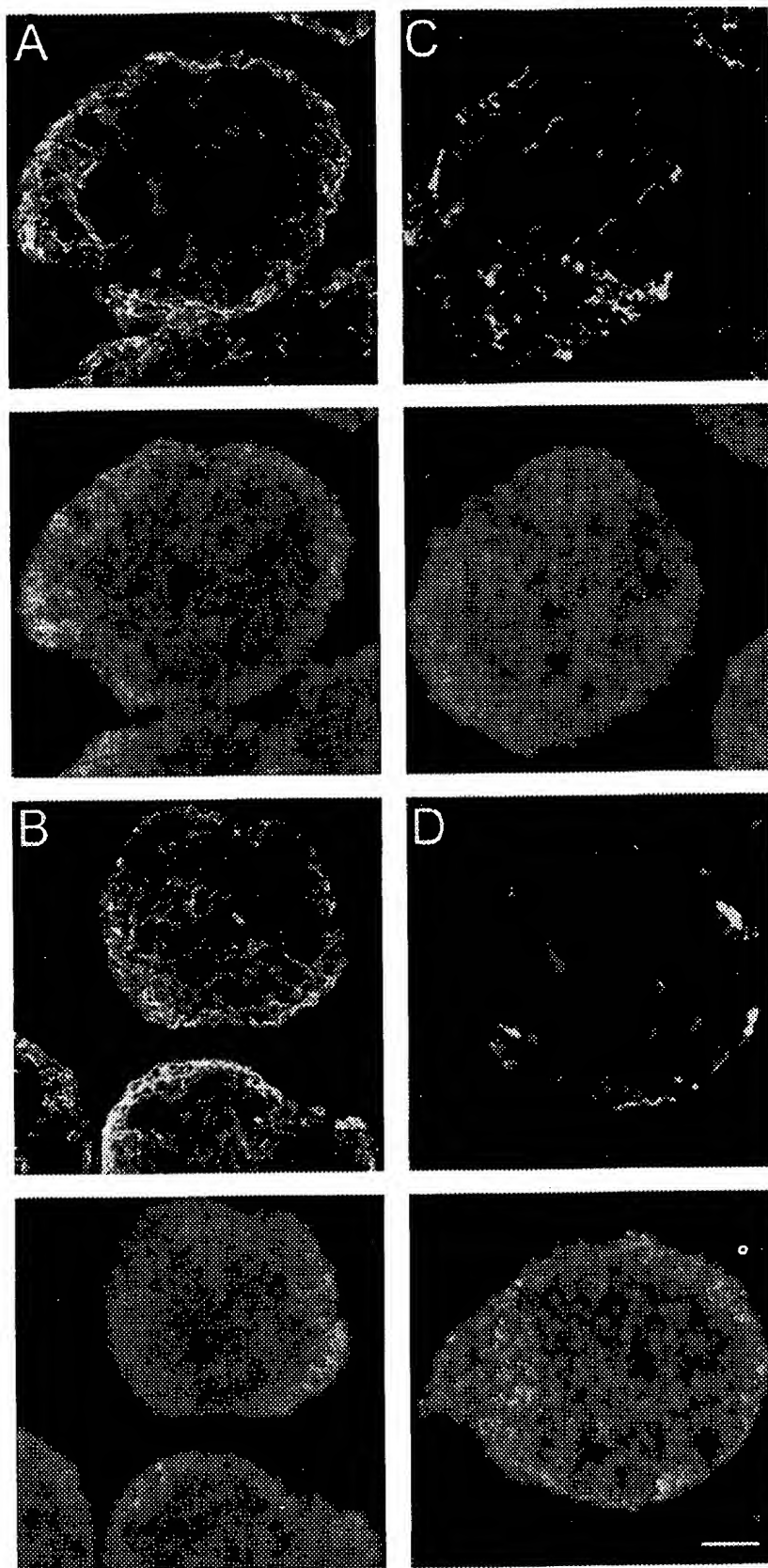


Figure 7 Bone morphogenetic protein-4 does not enhance cell proliferation or death. (A) Cell number was determined from total DNA content as a function of time for control and BMP-4-treated ES cells. Bars show the means \pm S.E.M. for three inductions as a percentage of the number of cells on day 0. Filled bars = control; open bars = BMP-4 (8 ng/mL); hatched bars = aggregates maintained in DMEM plus 20% bovine serum. Pairwise comparisons at each time point revealed no significant difference ($p > .05$) between the two conditions. (B) Quantile plots of BrdU incorporation in day 5 and day 6 aggregate sections for two inductions. Aggregates exposed to BMP-4 for 24 (day 5) or 48 (day 6) h were not significantly different ($p > .05$, Mann-Whitney rank sum test) from controls. (C) Quantile plots of TUNEL staining in cryostat sections of embryoid bodies fixed on days 5 and 6. There was no significant difference ($p > .05$, Mann-Whitney rank sum test, $n = 3$ inductions) between control aggregates and those treated from the end of day 4 with BMP-4.



that these flat, elongated cells were not immunofluorescent for markers of fully differentiated epidermal cells (keratin), or cells of mesodermal lineages, such as bone (osteopontin, *stro-1*), muscle (muscle-specific actin), and endothelial cells (MJ7/18, MECA-32). They were, however, strongly positive for vimentin (Fig. 5), an intermediate filament protein which is expressed by early-migrating mesodermal and mesenchymal cells (Klinowska et al., 1994), but also by immature astrocytes and radial glial cells (Hutchins and Casagrande, 1989), as well as other cell types (Osborn, 1983).

We also employed RT-PCR to test for expression of cell type-specific genes in control cultures and in cultures prepared from BMP-4-treated aggregates. As shown in Figure 6(A), *brachyury*, a gene expressed transiently in all mesodermal cells (Wilkinson et al., 1990; Herrmann et al., 1990), was detected in cultures from BMP-4-treated aggregates (three of six inductions) but not in control cultures. The reverse was true for *GluR6*, a kainate-type glutamate receptor subunit expressed in some neurons and astrocytes (Egebjerg et al., 1991). In addition, another marker of mesodermal lineage, *thx6* (Chapman et al., 1996), was selectively expressed in cultures of BMP-4-treated aggregates (two of four inductions) [Fig. 6(B)]. Collectively, these results suggest that BMP-4 promotes mesodermal differentiation while limiting the differentiation of neural cell types.

BMP-4 Does Not Alter Cell Proliferation or Apoptosis

In theory, BMP-4 might cause an apparent reduction in the proportion of neural cell types by stimulating proliferation of nonneural cells. Although we did not observe an obvious increase in aggregate size or number in BMP-4-treated cultures, we determined whether BMP-4-treated cultures showed any increase in overall cell number relative to control using an assay for total cellular DNA content (see Materials

and Methods). Cells from control and BMP-4-treated cultures were collected every other day from time zero through the eighth day of induction and their total DNA content was determined. There was no significant difference between control and BMP-4-treated ES cells at any time point [Fig. 7(A)]. Thus, BMP-4 does not reduce the percentage of neurons by causing a large increase in the number of nonneural cells.

We looked more closely at BMP-4 effects on proliferation using incorporation of bromodeoxyuridine (BrdU). ES cell aggregates were cultured for 4 days in NB+B27, then split into control and BMP-4-treated subcultures. Cultures were fixed at the end of day 5 or day 6 following a 1-h incubation with BrdU (3 μ g/mL). As shown in Figure 7(B), there was no significant difference in BrdU incorporation between control and BMP-4-treated aggregates on days 5 or 6. These results confirm that 24- to 48-h exposure to BMP-4 does not cause an increase in ES cell proliferation.

Bone morphogenetic proteins have been demonstrated to play a pro-apoptotic role in shaping the limb bud (Yokouchi et al., 1996) and in the development of the neural crest (Graham et al., 1994). In these systems, BMPs induce large populations of cells to die. To determine whether exposure to BMP-4 promotes apoptosis in ES cell neuronal precursors, we used a modified version of the TUNEL reaction (see Materials and Methods). Control aggregates and aggregates exposed to BMP-4 starting at the end of day 4 were processed for the TUNEL reaction on days 5 and 6 (24 and 48 h later). Consistent with previous work (Cocouvanis and Martin, 1995) which demonstrated a finite level of apoptosis during embryoid body maturation, we observed that approximately 25% of the cells in 5- and 6-day aggregates were positive for TUNEL staining; however, there was no significant difference between control and BMP-4-treated aggregates in the percentage of TUNEL-positive cells [Fig. 7(C)].

Figure 8 boc-aspartyl(OMe)-fluoromethylketone (BAF) does not enhance neural differentiation in control or BMP-4-treated aggregates. Aggregates were maintained in suspension in NB plus B27 (A,B) or in the presence of 8 ng/mL BMP-4 (days 5–8) (C,D). Half of the aggregates from each condition were incubated with 50 μ M BAF (days 5–8) (B,D). Immunofluorescence for TuJ1 is shown in sections of 8-day (A, B) and 13-day (C,D) aggregates. DAPI-stained nuclei are shown on the bottom, and immunofluorescence on the top of each lettered panel. Bar = 50 μ m. TUNEL labeling was performed in sections of aggregates sampled at the end of day 5 (24 h after application of BMP-4 and/or BAF). The median percentage of TUNEL-positive cells were: control = 23.3% (13.7, 37.4); control + BAF = 15.2% (9.7, 25.0); control plus BMP-4 = 23.4% (19.2, 28.8); control plus BMP-4 plus BAF = 10.6% (7.6, 22.0). The 25th and 75th percentiles are given in parentheses. BAF significantly reduced ($p < 0.05$, Mann-Whitney rank sum test) the percentage of TUNEL labeling relative to control and control plus BMP-4 conditions.

To test whether inhibition of apoptosis could alter the differentiation of neurons in control or BMP-4-treated aggregates, we examined the action of boc-aspartyl(OMe)-fluoromethylketone (BAF), a compound which reduces cell death by inhibiting members of the interleukin-1 β converting enzyme (ICE) family of proteases (Deshmukh et al., 1996). Incubation with BAF (30–50 μ M) reduced the per-

centage of TUNEL-positive cells by 35% and 54% in control and BMP-4-treated conditions, respectively. However, addition of BAF during BMP-4 treatment (days 5–8) did not increase the proportion of TuJ1-positive cells that were present on day 13 (Fig. 8). Taken together, these results indicate that apoptosis of neurons or neuronal precursors is not the mechanism by which BMP-4 inhibits neural phenotypes.

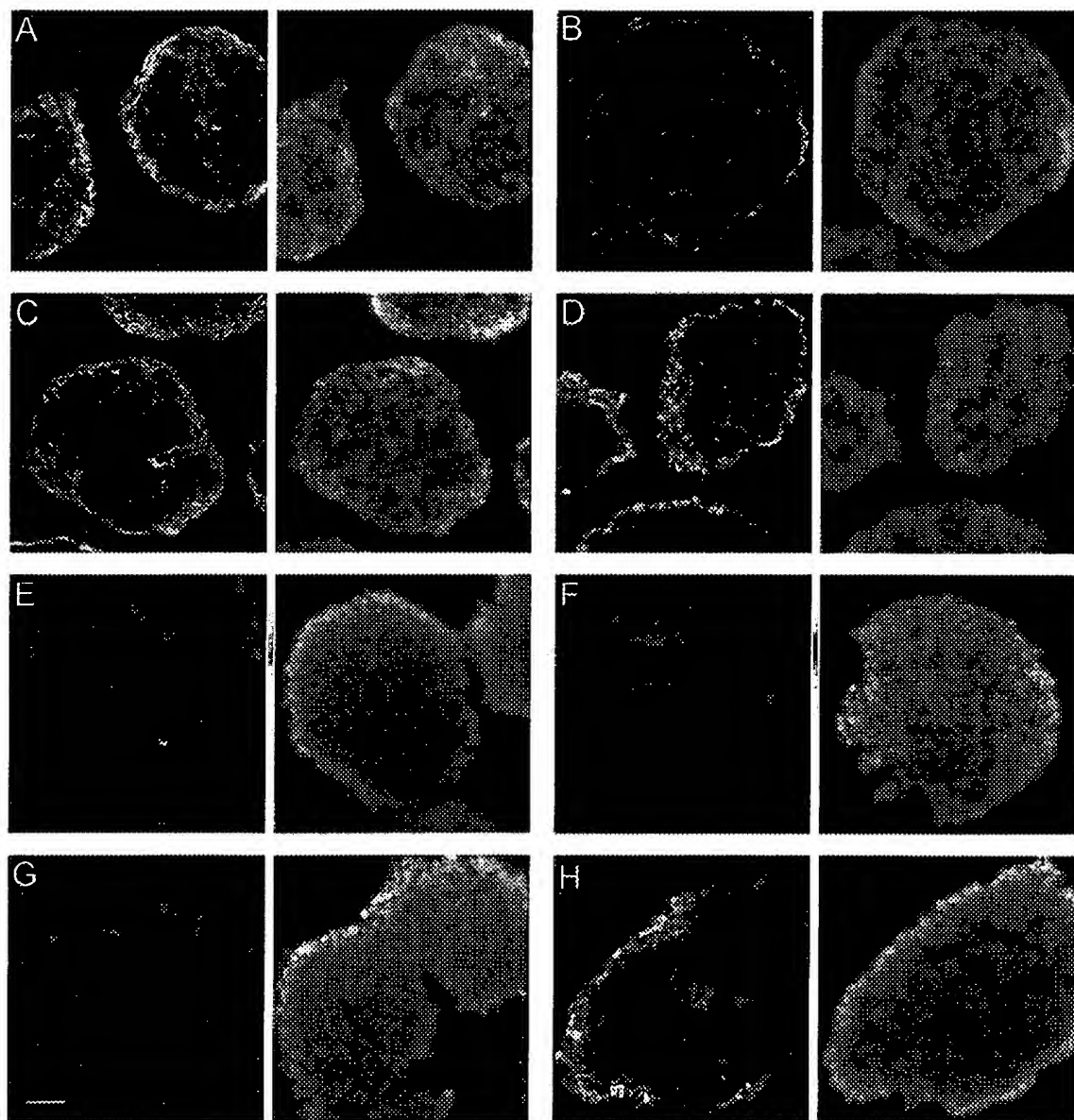


Figure 9 rhNoggin inhibits BMP-4 action. (A–D) Sections of 13-day aggregates stained for TuJ1. Aggregates were maintained in NB plus B27 for 13 days (A), or were treated from day 5 through 8 with 8 ng/mL BMP-4 (B), 160 ng/mL Noggin (C), or Noggin plus BMP-4 (D). (E–H) TuJ1 staining in sections of aggregates that were maintained for 8 days without retinyl acetate. Aggregates were kept in NB plus B27 (–) for 8 days (E) or were treated from day 5 through 8 with 160 ng/mL Noggin (F), 160 ng/mL Noggin Δ B2 (G), or 1 μ M RA (H). DAPI-stained nuclei are shown on the right, and immunofluorescence on the left of each lettered panel. Bar = 50 μ m.

Noggin Inhibits BMP-4 Action

The ability of a transplanted Spemann organizer to induce neural tissue has led to the discovery of several neuralizing proteins, including noggin (Lamb et al., 1993), chordin (Sasai et al., 1995), and follistatin (Hemmati-Brivanlou et al., 1994), which at early stages of development promote neurogenesis. Recent experiments have identified these proteins as BMP-4 antagonists (Zimmerman et al., 1996; Sasai et al., 1995; Fainsod et al., 1997). We tested whether treatment of ES cells with noggin could increase the level of neuronal differentiation under control conditions and whether it could antagonize the action of BMP-4 on ES cell neurogenesis. Exposure of ES cell aggregates to rhNoggin, either in the presence or absence of retinyl acetate, did not alter the level of TuJ1 expression (Fig. 9 and Table 2). However, coapplication of rhNoggin with BMP-4 resulted in a return of TuJ1 expression in 13-day aggregates to control levels (Fig. 9). In addition, aggregates exposed to a combination of rhNoggin and BMP-4 did not give rise to the flat, elongated, vimentin-positive cells which were observed following treatment with BMP-4 alone. These data indicate that the noggin used in our experiments is functional and that it does antagonize the action of BMP-4. However, the addition of exogenous noggin alone is insufficient to induce neuronal differentiation in mammalian ES cells (cf. Streit et al., 1998).

DISCUSSION

Totipotent ES cells can differentiate into a wide variety of cell types. *In vivo*, ES cells incorporated into a host blastula will populate the germ line as well as virtually every somatic cell population (Bradley et al., 1984). In addition, ES cells can be induced *in vitro* to acquire the phenotypes of specific differentiated cell populations, including hematopoietic cells, endothelial cells, muscle, and neurons (reviewed by Keller, 1995). Both *in vivo* and *in vitro*, the differentiation of ES cells is under the regulation of specific molecular control mechanisms. Previous work (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995) demonstrated that exposure of aggregates to retinoic acid triggers the efficient differentiation of ES cells into neurons and glia. In the present study, we have shown that the TGF β family member BMP-4 acts during a restricted time window to inhibit retinoid-mediated neural differentiation. Application of BMP-4 during the fifth through eighth day of induction overcame the neuralizing action of retinyl acetate or retinoic acid, whereas earlier or later exposure to BMP-4 had no significant effect on *in vitro* neurogenesis.

Table 2 Exposure to Noggin Alone Has Little Effect on Neuralization

Exposure to Noggin (days)		TuJ1 Expression	n
1-4	5-8		
Control (retinyl acetate)		+++	5
****	****	+++	2
	****	+++	3
Control (retinoid free)		+/-	5
****	****	+/-	2
	****	+/-	3

(Left) Exposure to 160 ng/mL rhNoggin during days 1-4 or 5-8 in suspension is indicated by asterisks. (Right) The level of TuJ1 expression is scored on the same qualitative scale used in Table 1; +/- indicates little or no neuralization [e.g., Fig. 9(E-G)]; +++ indicates significant neuralization [e.g., Fig. 9(A) or (H)]. n = number of inductions observed. For the first three conditions, aggregates were cultured in neurobasal medium plus B27 supplement that included retinyl acetate. For the last three conditions, B27 supplement which lacked retinyl acetate was used.

Previous work on P19 embryonal carcinoma cells also has demonstrated a reduction in neuralization with BMP-4 treatment (Glozak and Rogers, 1996; Hoodless and Hemmati-Brivanlou, 1997); however, in this case more than 90% of the cells underwent apoptosis within 4 days (Glozak and Rogers, 1998). By contrast, we observed no significant change in either ES cell number or cell death in BMP-4-treated versus control conditions. In addition to reducing neural differentiation, exposure to BMP-4 enhanced expression of mesodermal markers in mammalian ES cells (see also Johansson and Wiles, 1995; Vidricaire et al., 1994). Embryoid bodies that had been treated with BMP-4 gave rise to a distinct population of vimentin-positive nonneuronal cells and showed increased expression of *brachyury* (Herrmann et al., 1990) and *tbx6* (Chapman et al., 1996). Although *brachyury* and *tbx6* were not detected in all of the BMP-treated inductions, this may reflect their transient expression during the course of differentiation (Wilkinson et al., 1990; Chapman et al., 1996). In earlier work on ES cells in serum-containing cultures, Bain et al. (1996) used RNAase protection assays to demonstrate robust *brachyury* expression under conditions that promote the acquisition of fully differentiated mesodermal phenotypes. Johansson and Wiles (1995), however, in an RT-PCR study of serum-free ES cell differentiation, showed that the time course of *brachyury* expression depends on the concentration BMP-4 in the medium. In the present study, we analyzed each induction at a single time point between the fifth and seventh day after plating (9-11 days after the onset of BMP-4). Clearly, further work using

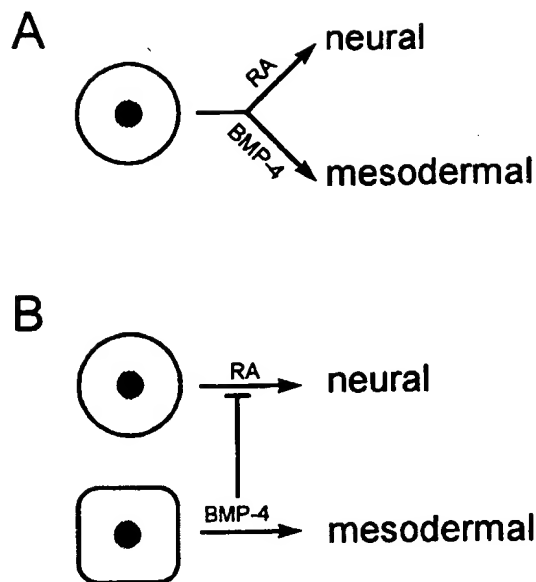


Figure 10 Two possible models for BMP-4 action on ES cells. (A) BMP-4 and RA compete at the level of individual cells to direct them toward either a mesodermal or neural fate, respectively. (B) BMP-4 and RA act on separate populations of cells: RA promotes neural induction of one population, while BMP-4 promotes mesodermal induction of another population and inhibits neural differentiation of the RA-responsive population.

more quantitative assays for mRNA will be needed to define all of the parameters that regulate expression of these genes during ES cell differentiation. However, the results collectively suggest that BMP-4 may instruct differentiating ES cells to change from a neural to a mesodermal fate.

Figure 10 presents two simple models that might explain the action of BMP-4 on differentiating ES cells. In the first, BMP-4 acts at the level of single cells to direct cell fate choice toward mesodermal and away from ectodermal (neural) cell fates [Fig. 10(A)]. In the second, BMP-4 acts on one population of cells, directing them toward mesodermal differentiation, while simultaneously inhibiting the retinoid mediated neuralization of a second population [Fig. 10(B)]. Further analysis of these early steps in differentiation would be greatly aided by discovery of better markers for ectodermal and mesodermal precursor cells. However, formal proof of either model, or of some other more complex interaction, will require analysis of ES cell differentiation at the level of single cells (Shah et al., 1996), an experiment which currently is not feasible because efficient neuralization requires a period of ES cell aggregation.

Our observation that BMP-4 enhances mesodermal differentiation is in line with previous work on ES

cells (Johansson and Wiles, 1995) and is consistent with the *in vivo* expression of BMP-4 very early in development. The results in Table I show that BMP-4 acts during the same 4-day period as RA (Bain et al., 1996), but our experiments did not determine the precise temporal relationship of BMP-4 and RA action. For example, mesoderm induction by BMP-4 may occur before RA can direct neural induction, thus preventing cells from ultimately becoming neurons. *In vivo*, the fate choice between mesoderm and ectoderm occurs at the time of gastrulation, before neuralization begins. In *Xenopus*, BMP-4 can promote the differentiation of mesoderm during gastrulation (Jones et al., 1996) or directly induce mesoderm as a heterodimer with other BMPs (Suzuki et al., 1997b). In addition, mouse embryos with homozygous null mutations in the *bmp-4* or *bmpr* genes exhibit severe defects in gastrulation and mesodermal differentiation (Winnier et al., 1995; Mishina et al., 1995). Thus, a major action of BMP-4 early in development is to promote the differentiation of mesoderm.

Other recent work on *Xenopus* animal cap ectoderm (Wilson and Hemmati-Brivanlou, 1995) has shown that BMP-4 can enhance the production of epidermis as it inhibits neuralization. Furthermore, a recent study of P19 embryonal carcinoma cells (Hoodless and Hemmati-Brivanlou, 1997) reported that incubation of monolayer cultures with BMP-4 increased the expression of epidermal cytokeratins. In contrast, we failed to obtain evidence for an increase in epidermal phenotypes following exposure of ES cells to BMP-4, either in aggregate cultures (Fig. 5) or in monolayers (not shown). This difference may reflect changes in the downstream targets of BMP-4's action during the course of development, with ES cells displaying an earlier cell fate decision than P19 cells or *Xenopus* ectoderm. Put another way, the promotion of distinct phenotypes by BMP-4 may be owing to differences in developmental potential between totipotent ES cells and the more restricted fate choices of embryonal carcinoma cells (Bradley et al., 1984) or *Xenopus* ectoderm (Sasai and DeRobertis, 1997).

In *Xenopus*, the blockade of BMP-4 signaling by noggin, chordin, or follistatin is sufficient to neuralize the animal cap ectoderm (Wilson et al., 1997; Sasai et al., 1995; Fainsod et al., 1997), an observation which has led to the proposal that neural differentiation may be the default fate of early ectoderm (Hemmati-Brivanlou and Melton, 1997). Much less is known about the early steps in mammalian development; however, two recent papers have suggested that blockade of BMP signalling can promote neuralization of P19 embryonal carcinoma cells (Fainsod et al., 1997; Hoodless and Hemmati-Brivanlou, 1997). In

one study (Fainsod et al., 1997), cells expressing neurofilament and neural cell adhesion molecule immunoreactivity, as well as GFAP-positive cells, were observed following stable transfection of P19 cells with *follistatin*. Although the proportion of cells with neural phenotypes was not determined in this study (Fainsod et al., 1997), aggregation or exposure to exogenous RA was not required; the P19 monolayers were simply maintained in 10% serum and treated with a mitotic inhibitor. In the second study (Hoodless and Hemmati-Brivanlou, 1997), stable transfection with a truncated activin type II receptor caused approximately 2% of P19 cells to acquire neuronal morphology and express neurofilament proteins. This truncated receptor inhibits signaling by several different TGF- β family members, including BMPs and activins (Hoodless and Hemmati-Brivanlou, 1997). In contrast to RA-mediated neuralization of P19 and ES cells, or transfection with *follistatin* (see above), GFAP-positive astrocytes were not observed in cultures of P19 cells transfected with the truncated activin receptor (Hoodless and Hemmati-Brivanlou, 1997).

In the present study, we demonstrate the window of competence for BMP-4 to inhibit neuronal differentiation of ES cells. Our results suggest that BMP-4 may not be the only factor responsible for regulating this process. Incubation with the BMP-4 antagonist noggin was able to counteract the effect of exogenous BMP-4; however, exogenous noggin alone did not enhance neuronal differentiation in either the presence or absence of retinoids (Fig. 9 and Table 2). These results indicate that totipotent mammalian ES cells do not become neurons by default. In future experiments, it will be important to examine additional markers for very early neuroectoderm, such as *Sox* gene expression (Pevny et al., 1998), to see whether exposure to BMP antagonists might allow for rudimentary differentiation but be insufficient for generation of neurons and glia. Further work also is needed to test whether neuralization might proceed more efficiently in ES cells that were engineered to overexpress noggin, or some other BMP antagonist. In this regard, it is interesting to note that a recent study in chick (Streit et al., 1998) has demonstrated that ectopic expression of *chordin* is insufficient to induce expression of early neural markers in regions of the epiblast which can be neuralized by a Hensen's node graft. Taken together, these seemingly conflicting results emphasize that subtle but important differences exist among vertebrates in the roles of conserved signaling molecules and in the competence of specific cell types to respond to these signals. Moreover, they point to the necessity of multiple experimental models for studying neuralization.

In summary, our data demonstrate that BMP-4 can regulate the differentiation of mammalian ES cells *in vitro*. Exposure to BMP-4 inhibits neuralization and promotes expression of markers that are consistent with mesodermal differentiation. This action of BMP-4 on differentiating ES cells is likely to correspond to its very early regulation of the fate choice between ectoderm and mesoderm *in vivo*. The ability to control the differentiation of large numbers of ES cells *in vitro* makes this an attractive system for future work on the signaling molecules downstream of BMP-4, including the BMP receptors Smads and *msx*.

Note added in proof: A recent study of ES cells by Rohwedel et al. (Dev Biol 201:167–184, 1998) indicates that exposure to BMP-4 accelerates the expression of brachyury and delays expression of the neuronal gene *wnt-1*.

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